식물조직배양학회지 제27권 제5호
Korean J. Plant Tissue Culture
Vol. 27, No. 5, 415~421 (2000)

Trehalose Metabolism: Gate to Stress Signaling and Seed Development in Plant?

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ABSTRACT The disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is found in variety of organisms that are able to withstand almost complete desiccation. In order to identify the function of trehalose in plants, we isolated Arabidopsis trehalase (AtTRE) gene that encodes the enzyme able to hydrolyze trehalose to glucose, and trehalose-6-phosphate synthase isolog, TPS3 gene by RT-PCR. The AtTRE had the substrate specificity to hydrolyze only trehalose, and a broad pH range of enzyme activity. The AtTRE promoter/GUS reporter gene was expressed in cotyledons, mature leaf tissues including guard cells, and developing siliques. The GUS expression driven by AtTPS3 promoter was significant in root tissues, and the level of GUS activity was much higher than that of the pBI121 control seedlings. The knockout of AtTPS3 gene in Arabidopsis resulted in the retarded root development, whereas the overexpression of AtTPS3 increased the root elongation in the presence of sucrose in MS medium. Possible functions of AtTRE and AtTPS3 in plant will be discussed. In addition, ectopic expression of yeast TPS1 driven by the inducible promoters in tobacco and potato conferred the plants on the drought and freezing tolerances.

Introduction

Trehalose is a non-reducing disaccharide present in many organisms as fungi, invertebrates, yeasts, and bacteria, and has a wide range of functions. In yeast, trehalose highly accumulates in response to stresses such as exposure to heat, and desiccation, proposing that trehalose functions both as a stress protectant and the storage compound. In addition, it has been known as a useful food stabilizer and an additive in cosmetics and pharmaceuticals (Goddijn and Kun 1999; Jorge et al. 1997; Porchia et al. 1999; Singer and Lindquist 1998). Presence of trehalose in plants was restricted in a few pteridophytes (ferns), and resurrection plants such as *Selaginella lepidophylla* and *Myrothamnus flabellifolia*. However, recently consecutive experiments showed ubiquitous presence of trehalose in higher plants with indefinite function (Blazquez et al. 1998; Vogel et al. 1998; Zentella et al. 1999).

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The biosynthesis of trehalose comprises of two steps: the formation of trehalose-6-phosphate out of UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS), and the subsequent dephosphorylation of trehalose-6-phosphate into trehalose by trehalose-6- phosphatase (TPP). In *E.coli*, OtsA and OtsB genes encode the TPS and TPP enzymes, respectively. In contrast, the formation of trehalose in yeast requires the enzyme complex consist of Tps1p, Tps2p, Tsl1p, and Tps3p. Enzyme Tps1p functions as TPS whereas Tps2p does as TPP (Reinders et al. 1997; Bell et al. 1998). In higher plants, several TPS homologs or ESTs have been either cloned from Arabidopsis, resurrection plant, rice, and cotton, or found in DNA databases. Both *Arabidopsis* TPS1 (AtTPS1) and *Selaginella lepidophylla* TPS1 (SlTPS1) could restore the growth or the thermotolerance of the yeast *tps1* mutant that was unable to grow in glucose as the sole carbon source (Blazquez et al. 1998; Zentella et al. 1999). However, there are many questions need to answer what the exact function of these TPS genes in plant systems.

Trehalase is a specific enzyme that hydrolyses trehalose to yield two molecules of glucose. Two types of

trehalase have been identified: a neutral trehalase and an acid trehalase. The neutral trehalase with a pH optimum at 7 locates in the cytosol and is activated by cAMP-dependent phosphorylation. NTH1 and NTH2 genes encode the neutral trehalase Nth1p and Nth2p, respectively. A putative trehalase Nth2p has no detectable trehalase activity. The acid trehalase is a vacuolar protein with a pH optimum at 4.5 (Jorge et al. 1997; Singer and Lindquist 1998). The biological role of trehalases has remained unclear yet, however it has been speculated that the neutral trehalase mobilizes a cytosolic trehalose and that the acid trehalase hydrolyses extracellular trehalose (Jorge et al. 1997). Have the greater length of discovery of trehalase activity in higher plants, the trehalase clones were recently isolated in potato, *Glycine max*, and *Arabidopsis* (Aeschbacher et al. 1999; Muller et al. 1999). In higher plants, however, little is known about the function of trehalase at biochemical and biological levels. We isolated *Arabidopsis* trehalase and TPS3 clones by the RT-PCR method, and show that their tissue localizations by GUS gene expression driven by promoters as well as the western analysis. Biochemical and biological functions of both trehalase and TPS3 will be also discussed.

Materials and Methods

RT-PCR of Arabidopsis trehalase and TPS3 clones

Total RNA was isolated from *Arabidopsis* seedlings and cDNA was synthesized as following condition. 5 μg of total RNA was reverse transcribed in 20 μl of reaction mixture containing 4 μl of 5 M-MLV RT buffer, 2 μl of 10 mM dNTPs, 1 μl of 100 mM oligo-dT primer, 0.5 μl of RNasin (40 units/μl, Promega, Madison, USA), and 1 μl of M-MLV RT (200 units/μl, Promega, Madison, USA). The reaction mixture was incubated at 65°C for 3 min without RNasin and M-MLV RT, and subsequently incubated at 42°C for 60 min after addition of RNasin and M-MLV RT, followed by incubation at 52°C for 30 min. The remaining RNA was inactivated by incubation at 95°C for 10 minutes. Gene specific primers (AraTre5: 5'-atgttggactcggacacaga-3'; AraTre3: 5'-ctaggcttcaatgctaagatg-3') were designed based on *Arabidopsis* BAC T19F06 genomic sequence containing trehalase precursor isolog. The *Arabidopsis* trehalase was amplified using these primers with 1 μl of cDNA preparation. The PCR was performed for 30 cycles. The PCR product was cloned into pGEM-T easy vector (Promega, Madison, USA), and the nucleotide sequences were verified by sequencing using ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, USA). For the production of recombinant protein, the trehalase cDNA was inserted into *E. coli* expression vector pRSET (Invitrogen, San Diego, USA), which was designated as pLES98060.

Expression and purification of recombinant trehalase protein

The pLES98060 was introduced into *E. coli* BL21 (DE3) pLysS. Expression and purification of Recombinant protein was expressed and purified as the manufacturer's guide. In brief, ampicillin resistance transformed clone was incubated to $Abs_{600} \cong 0.6$ in LB broth medium, and then IPTG was added to 1 mM final concentration. After further incubation at 37°C for 4 hours or 25°C for overnight, the cells were harvested by centrifugation at 3,000 rpm for 15 min. The cells were resuspended in a lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole). Soluble fraction was collected by sonication of the suspension and centrifugation, and was analyzed by SDS-PAGE. The His-tagged protein was purified from soluble fraction by step gradient elution. The purity of elutes was checked by SDS-PAGE analysis.

Assay of trehalase activity

With trehalase purified from porcine kidney (Sigma, St. Louis, USA) as a standard, activity of Arabidopsis trehalase was tested. The elutes or standard trehalase was added in 10 mM α,α-trehalose and 50 mM MES (K^+), pH 6.3, and the mixture was incubated at 37°C for 30 min. Boiling for 3 min stopped the reaction and the glucose content in this mixture was measured with the glucose oxidase-peroxidase test kit (Sigma, St. Louis, USA) for detection of the glucose produced by hydrolysis of trehalose.

PCR cloning of Arabidopsis trehalase and TPS3 promoters

Based on *Arabidopsis* genomic sequence data in GenBank, each primer sets were designed for amplification of trehalase or TPS3 promoter regions. The PCR reaction contained 500ng of genomic DNA, 5ul of 10x Taq polymerase buffer, 1mM of $MgCl_2$, 200 μM of dNTPs, 20 pmole of each primer, and 5U of Taq polymerase. The reaction was hot started and amplified as the following conditions: 1 cycle of 95°C for 5 minute as post-denaturation; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute; 1 cycle of 72°C for 10 minutes as post-extension. The amplified product was gel-purified using QIAquick gel extraction kit (Qiagen) and subsequently cloned into the pGEM-T easy vector (Promega) by the manufacturer's guide. The nucleotide sequences were verified by sequencing using ABI Prism 310 Genetic Analyzer. For the promoter-GUS reporter gene constructs, the insert from pGEM-T easy vector was subcloned into the binary vector pBI101.

Generation of transgenic plants

The plasmids containing the promoter/GUS gene fusion, sense or antisense of AtTPS3 cDNA were transformed into *Agrobacterium tumefaciens* strain GV1301 and transferred to *Arabidopsis thaliana* (L.) Heynh., ecotype Col-0 via vacuum infiltrated transformation. For a selection of kanamycin resistant plants, F_1 seeds were surface sterilized with 70% ethanol followed by 50% household bleach with Tween 20, and then washed four times in sterilized water. Seedlings were grown in Murashige and Skoog (MS) medium agar plates containing 1% sucrose and 50 μg/ml kanamycin and placed in either a vertical or horizontal position. Plants were grown at 22 to 24°C under 16 hours of light period. The transgenic lines of one copy insertion were verified by kanamycin segregation ratio with F_2 seedlings, and their homozygote progenies were assayed.

GUS activity assay of transformed Arabidopsis

Transformed Arabidopsis plants were subjected into GUS activity assay. The full grown Arabidopsis leaves were detached and soaked into assay solution containing following components: 100mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1 % Triton X-100, 0.3% 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcA). After overnight incubation at 37°C the leaves were transferred into 100% EtOH solution to decolorize the leaf chlorophyll. GUS stained leaves were examined under the microscopy.

For the biochemical GUS assay, sample tissues were homogenized in GUS extraction buffer (50mM $NaPO_4$, pH 7.0, 10mM EDTA, 0.1% sarkosyl, 0.1% Triton X-100, 10mM β-mercaptoethanol). Samples were centrifuged for 10 min at 4°C, and the supernatant was used for the GUS assay. For fluorimetric reactions, duplicate reactions carried out by adding 10mM 4-methylumbelliferyl β-D-glucuronide (4-MUG) to 1mM concentration and incubating at 37°C. One reaction was terminated at 5 min as control, and the second at 65 min with the addition of 0.2 M Na_2CO_3. Fluorescence was measured on a TD-700 fluorometer (excitation wavelength = 365nm, photeodetector wavelength = 460nm) after dilution with 0.2 M Na_2CO_3. The protein content of the samples was determined using a protein assay kit (BioRad) following the manufacturer's protocol. For histochemical GUS analysis, seedlings were immersed in the GUS reaction buffer (2mM 5-bromo-4-chloro-3-indolyl-β-D glucuronic acid (X-Gluc), 1% dimethylformamide, 0.1mM potassium ferricyanide, 0.1mM postassium ferrocyanide, 1mM EDTA, 50mM $NaPO_4$, pH 7.0) followed by brief vacuum infiltration. Tissues were incubated at 37°C for 4-16 hours. After incubation, seedlings were cleared in 70% ethanol to remove chlorophyll for better visualization, and photographed with digital camera attached to the stereo microscopy (Olympus SZX) with either dark-field or bright-field.

Results and Discussion

Characteristics of Arabidopsis trehalase enzyme

Arabidopsis trehalase (AtTRE) was obtained by the RT-PCR method. The deduced amino acid sequence of trehalase showed a highly homology to trehalases of both yeast and *E. coli*. In order to characterize the plant trehalase enzyme, the AtTRE gene was expressed in *E.coli* and determined if it could hydrolyze trehalose.

Purified recombinant protein was assayed with various substrates (Table 1). As we expected the AtTRE hydrolyzed only trehalose disaccharide, not other substrates. Two types of trehalase identified in other organisms were classified based on their optimal activity at pH level. We next determined the optimal pH of AtTRE ranging from pH3.5 to pH8.0 (Table 2). In a broad range of pH buffer MES, the AtTRE enzyme was active from acid to neutral pH ranges. Similar results were also obtained in both sodium citrate and citrate phosphate buffers of which pH zones were pH4.5 to pH8.0 or pH4.5 to pH7.0, respectively. These results showed that unlike the trehalases of yeast, fungi, and bacteria the AtTRE had a broad range of pH optimal activity. Trehalase so far identified in higher plants has been known a single copy gene. This might explain why AtTRE enzyme has the broad pH range that covers most of pH zones in plant cells. Preliminary results of immunolocalization showed that trehalase presents in the cytosol (data not shown). However, more detailed study for organelle location is necessary. Table 3 shows that the activity of AtTRE was increasing as the temperature increased to 50°C, indicating that the expression of trehalase in plants is probably induced by high temperature exposure.

Expression pattern of Arabidopsis trehalase promoter

In order to identify the localization and regulatory function of 5'upstream region, a series of deletions of the trehalase were constructed, fused to a GUS reporter gene cassette, and used for *Arabidopsis* transformation. In young seedling harboring 2 kb promoter of AtTRE gene, the GUS expression was restricted in cotyledons (Figure 1A). The GUS gene expression was detected in mature leaf tissues including guard cells, but in only guard cells of stem (Figure 1B and C). Interestingly, the GUS activity could detect during the seed development. For example, the GUS expression was first observed in the style of young silique, and moved to the transmitting tissues (Figure 1D and E). During the late phase of seed maturation, the GUS activity was specifically localized in the central replum and funiculus through which seeds are attached, and its expression level gradually

Table 1. Substrate specificity of Arabidopsis trehlase.

Substrate*	A540	Glucose Amount (μg)
Glucose	1.060	50.00
Trehalose Control	0.002	-
Trehalose	1.154	54.34
Sucrose	0.211	0.00
Maltose	0.053	0.00
Lactose	0.006	0.00
Cellobiose	0.020	0.00
Melibiose	0.017	0.00

*50 μg of all substrates has been used for enzyme reaction.

Table 3. Optimal temperature of Arabidopsis trehalase*.

Temperature	Glucose Amounts (μg)
25°C	26.5
30°C	34.5
37°C	49.9
40°C	61.2
45°C	85.5
50°C	96.9

*50 μg of trehalose has been used as a substrate for enzyme reaction.

Table 2. Optimal pH range of Arabidopsis trehalase*.

pH	Glucose Amount (μg)		
	MES buffer	Sodium Citrate buffer	Citrate Phosphate buffer
Reagent Blank	-	-	-
Glucose, 50 μg	50	50	50
pH 3.5	67.4	NA	NA
pH 4.0	65.8	NA	NA
pH 4.5	62.6	45	44
pH 5.0	61.4	46	40.6
pH 5.5	50.2	46.8	39.4
pH 6.0	51.6	44.9	43.1
pH 6.5	53.4	44.4	47.2
pH 7.0	53.9	41.7	49.1
pH 7.5	56.7	44.6	NA
pH 8.0	60.4	43.8	NA

*50 μg of trehalose has been used as a substrate for enzyme reaction. NA, not assayed.

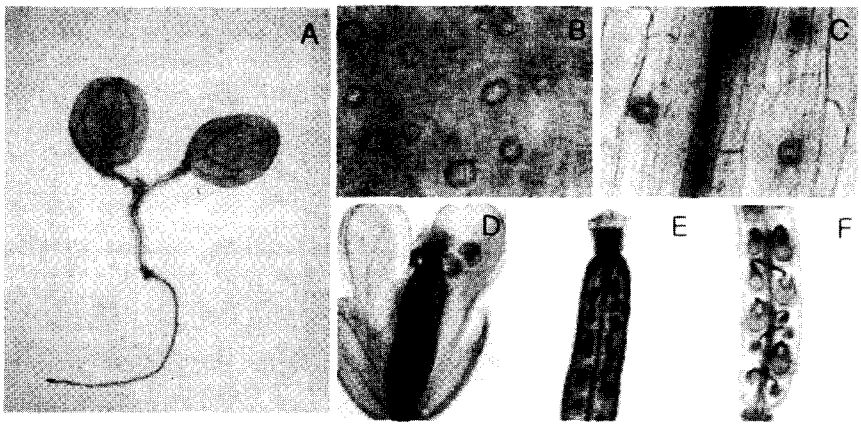

Figure 1. GUS expression driven by Arabidopsis trehalase promoter. Transgenic plant contains 2 kb size of AtTRE promoter fused to the GUS reporter gene. Single copy gene baring progenies were selected and stained in X-Gluc solution for overnight. The GUS expression was observed in cotyledons of 5 day-old seedling (A), in guard cells of mature leaf (B), in guard cells of mature stem (C), in the style of young silique (D), in the transmitting tissues (E), in the replum and funiculus (F).

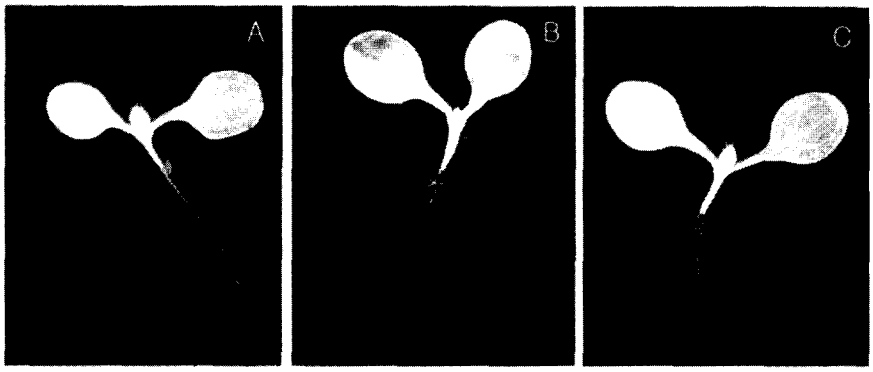

Figure 2. Strong and constitutive expression of AtTPS3 in roots. Transgenic plants are harboring CaMV35S promoter (A), 1 kb (B) or 2 kb (C) of AtTPS3 promoter/GUS fusion construct. Single copy progeny from each transgenic plant was selected and assayed. GUS staining was performed only 3 hours at 37°C to compare the GUS activity in roots.

decreased (Figure 1F). Matured embryos showed the GUS activity in embryonic root, and its expression continued but restricted to root rip area as germinated (data not shown). This is the first observation of that plant trehalase locates in siliques and maturing embryos. The GUS expression was reduced, as the promoter length decreases to 500 bp without changing the expression profile. This indicates that the 5' upstream region between 2 kb and 0.5 kb contains all necessary cis-acting elements of tissue expression and positive enhance of expression (data not shown).

We next determined whether there is a correlation between trehalose and its hydrolyzing enzyme trehalase by determining of the level of GUS expression induced by various environmental changes such as drought, salt, and exogenous treatment of abscisic acid. No change of the GUS expression was observed (data not shown). Therefore, at least the expression of trehalase is not correlated to the environmental stress such as drought and salinity. Recently, it has been emerged of new insights that trehalose functions as regulators of plant growth and development (Goddijn and Smeekens 1998; Vogel et al. 1998; Wingler et al. 2000). Taken together, these results suggest that trehalose might involve in seed development as an energy source of which glucose is the major carbohydrate that is recruited by trehalase, rather than stress tolerance.

Localization of Arabidopsis TPS3 gene

We found several AtTPS1 isologs in Arabidopsis genomic sequence data by the blast search using either partial or full sequences of AtTPS1. Ten TPS genes or isologs have been so far identified, and named as TPS2 through TPS10 according to the date of submission. We have cloned nine TPS isologs by RT-PCR or cDNA library screening, and also were amplified their promoters from genomic DNA using gene specific primers designed based on the sequence information. Among them we emphasized on AtTPS3 gene in this work. The deduced amino acid sequence of TPS3 was quite unique compared to AtTPS1 both at protein level and in genomic structure. In order to identify the tissue localization of AtTPS3, AtTPS3 promoter was fused to the GUS reporter gene, and the GUS expression profile were determined in developing seedlings. In 5 day-old seedlings, the expression of GUS gene driven by 1kb size of AtTPS3 promoter was highly accumulated in root tissues compared to that of 2 kb promoter (Figure 2B and C). Furthermore, the expression level of 1kb promoter

was much higher than that of the pBI121 control seedling (Figure 2A). In the case of 1 kb promoter, the GUS expression was also appeared in cotyledons. The level of GUS expression was dispersed and increased to all tissues, as the seedlings were mature.

Involvement of Arabidopsis TPS3 gene in root development

Ectopic expression of yeast TPS1 in tobacco or potato improves drought tolerance (Romero et al. 1997; Yeo et al. 2000). In order to elucidate the function of AtTPS3 in plants, transgenic *Arabidopsis* plants expressing antisense or sense of AtTPS3 cDNAs were generated. Figure 3 showed the phenotype of transgenic seedlings grown on the MS medium plate positioned in vertical. The MS medium contained 25mM sucrose as a carbohydrate supplement. Compared to the control seedlings containing one copy of pBI121 construct, the overexpression of AtTPS3 seedlings showed an increased growth rate of root development.

In contrast, the reduction of AtTPS3 in plant resulted in a retardation of root growth. We further determined the effect of various disaccharides on the root development (Figure 3D). The presence of trehalose in the MS medium resulted in significant slow root development in all transgenic seedlings including the control. With similar result was obtained by Wingler et al (2000), this result indicates that exogenous trehalose has a deleterious effect on the plant growth. The control seedlings grown on both sucrose and maltose containing medium appeared normal (Figure 3A). As described above, however, either overexpression or knockout of AtTPS3 transgenic lines showed an altered root growth rate in the presence of sucrose or maltose (Figure 3D). Root length of knockout seedlings grown on sucrose medium was about 2.3 and 4.8 folds shorter than that of the control and overexpression seedlings, respectively. Morever, this abnormal change of phenotype seems be confined in ground tissues, not in green tissues. Trehalose accumulation in plants was enhanced by addition of trehalase inhibitors including validamycin A and deoynojirimycin (Goddijn et al. 1997; Muller et al. 1995). To test whether the trehalase inhibitor affects the root growth rate, 10 μM of deoxynojirimycin was added to the MS medium. There was not effect on the root elongation of all transgenic seedlings. These data suggest that AtTPS3 gene may involve in root development. However, it is unclear yet whether the expression of AtTPS3 is correlated to the trehalose synthesis as a stress tolerant or the trehalase

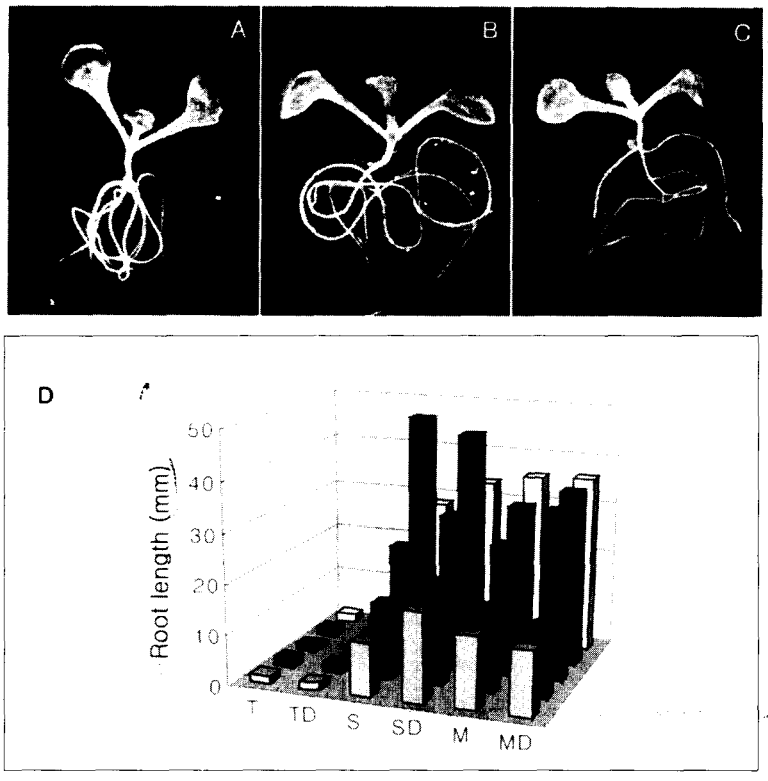

Figure 3. Developmental changes of root by overexpression and antisense of AtTPS3. Transgenic seedlings harboring CaMV35S (A), overexpression of AtTPS3 (B), and antisense of AtTPS3 (C) were grown on the MS medium with 25 mM of sucrose for 7 days. The plates were positioned in vertical for easy look of the root phenotype. (D) Seven day-old seedlings grown on 25 mM of trehalose, sucrose, and maltose in the presence or absence of 10 μM of deoxynojirimycin. Orange bars represent antisense line 1-1; blue, antisense line 23-2; red, sense line 9-5; green, sense line 15-2; yellow, pBI121.

expression in terms of the seed/plant development.

In spite of the known functions associated with stress tolerance in *E. coli* and yeast, fundamental function of trehalose-6-phosphate synthase in higher plants is indeed unclear. Much of our understanding of plant TPSs can be emerged by several approaches: 1) biochemical analysis using recombinant protein of TPS isoforms. 2) analysis of T-DNA knockout *Arabidopsis* mutant, together with antisense knockout transgenic plants. For this approach, it is kept in mind that the antisense knockout should be a gene-specific because of many TPS isoforms. 3) study of functional correlation between trehalase and TPS using the double direct knockout/overexpression mutants.

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